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# Nuclear Factor-*kappa* B is Not Up-regulated in Rat Pulmonary Tissue Following Sulfur Mustard (HD) Exposure

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**14. ABSTRACT**
Vesicants, such as sulfur mustard (HD), pose an insidious threat to the respiratory tract. Exposure causes acute lung injury characteristic of an inflammatory response. Nuclear Factor-*KAPPA* B (NF-□B) is a redox sensitive transcription factor important in regulating genes involved in the inflammation. This study evaluated levels of NF-□B following exposure to HD. Male rats were euthanized at 3, 6, or 24 hr following intravenous HD or isopropanol (IPA controls) exposure. Nuclear protein samples extracted from pulmonary tissue were analyzed using an electrophoretic mobility shift assay. Bronchoalveolar lavage fluid (BALF) protein concentrations and lactate dehydrogenase (LDH) were determined to indicate lung injury. NF-□B in HD lungs was not detected at a level significantly above that found in IPA samples. LDH was higher than in the IPA-treated group, $p<.013$, at 24 h. Protein concentrations were higher at 24 h compared with 3 and 6 h, $p<.05$. In conclusion, i.v. HD causes lung injury at 24 h as measured by BALF protein and LDH low-level inflammatory response related to time but not to dose. This model may be useful in developing anti-inflammatory treatments against HD exposure. These data suggest that alterations in NF-□B may not play a role in HD-induced lung injury in rodents intravenously exposed to HD.

**15. SUBJECT TERMS**
nuclear factor-*KAPPA* B; sulfur mustard; HD; pulmonary tissue; electrophoretic mobility shift assay, bronchoalveolar lavage fluid, lactate dehydrogenase, protein

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# INTRODUCTION

The known carcinogen and chemical warfare agent sulfur mustard [(bis-(2-chloroethyl)sulfide; HD)] is a lipid soluble, alkylating agent that is preferentially cytotoxic to cells with a high mitotic index. Large, fluid-filled blisters, characterized by dermal/epidermal separation, occur in humans following percutaneous exposure [1, 2]. Inhalation exposure causes toxicity to bronchial epithelial cells that can, if severe enough, lead to bronchopneumonia and death [3, 4]. HD is a highly reactive compound that is unstable in biological fluids, making it difficult to quantify the dose received deep in the lungs after an inhalation exposure. Previous investigators found that inhalation exposure to HD leads to clinical manifestations that are found primarily in the upper airway passages [5]. Literature references indicate that intravenous (i.v.) and subcutaneous HD exposures of rodents can be used to study pulmonary toxicity, thus circumventing the need for generating HD vapor for inhalation [6-8].

Although the exact mechanism of HD-induced tissue damage is not fully understood, HD exposure has been shown to result in the marked influx of inflammatory mediators [9-11]. Data in the literature demonstrate that subcutaneous injection of the monofunctional HD analog butyl 2-chloroethyl sulfide induces free radical-mediated oxidative pulmonary damage in mice [8]. Acute pulmonary inflammation involves the initiation of an inflammatory cascade that leads to an influx of cellular mediators responsible for increased vascular permeability and additional damage through the release of reactive oxygen species (ROS), proteases, and additional inflammatory mediators. ROS have been shown to initiate an inflammatory cascade through NF-κB activation [12, 13]. Since the effects of acute pulmonary inflammation are in part initiated through ROS, inflammation following exposure to HD may also be mediated through NF-κB. To study the relationship between NF-κB and oxidative tissue damage, studies were conducted to assay NF-κB in pulmonary tissue from animals following intravenous exposure to HD under time- and dose-dependent experimental circumstances.

NF-κB is a ubiquitous transcription factor of particular importance in the early amplification of an inflammatory response. NF-κB is normally present in the cell as inactive cytosolic complexes of NF-κB and the inhibitory protein called I-κB. Extracellular signals target the NF-κB:I-κB complexes, leading to a phosphorylation-dependent proteolytic degradation of I-κB. Widely thought to be activated by oxidative stress, NF-κB is released to translocate to the nucleus where it can bind to the promotor region of genes encoding various inflammatory mediators (e.g., chemokines and cytokines). The purpose of this study was to test the following hypotheses. Is there a direct effect on the lung following HD administration? Is oxidative stress involved in HD-induced lung injury? Is activation of transcription factors, such as NF-κB, an event associated with vesicating doses of HD? If so, then the biochemical events leading to activation may provide insight into the development of new therapeutic strategies to minimize HD-induced pulmonary toxicity.

# METHODS

Adenosine 5'-triphosphate [Ω -$^{32}$P] was purchased from NEN-Dupont Life Sciences Products, Boston, MA, USA. T4 polynucleotide kinase enzyme, oligonucleotides corresponding to the transcription factors AP-2 and NF-κB, and HeLa Nuclear Extract were purchased from Promega Corporation, Madison, WI, USA. Poly (dI-dC)•Poly (dI-dC) was purchased from

Pharmacia Biotech, Piscataway, NJ, USA. Dounce homogenizers were purchased from Daigger Company, Lincolnshire, IL, USA. G-25 Sephadex spin columns were purchased from Worthington Biochemical Company, Freehold, NJ, USA. BSA protein standards and bicinchoninic acid (BCA) assay reagents were purchased from Pierce Chemical Company Rockford, IL, USA. Mini-gel electrophoresis equipment, GS-700 Imaging Densitometer, Multi Analyst™ software, vacuum gel drying apparatus, cellophane sheets, filter paper, tris-borate-EDTA buffer, bromophenol blue dye, and polyacrylamide gels were purchased from Bio Rad Hercules, CA, USA. Unless otherwise indicated, all other chemicals were purchased from Sigma Chemical Company, St. Louis, MO, USA. The scintillation counter, model LS-3801, was manufactured by Beckman Coulter Corporation, Fullerton, CA, USA. Protease inhibitors were purchased from Promega Corporation, Madison, WI, USA (dithiothreitol [DTT]) and from Sigma Chemical Company, St. Louis, MO, USA (aprotinin, pepstatin A, leupeptin, and phenylmethylsulfonyl fluoride [PMSF]).

*HD Exposure.* HD exposure methodology closely followed that described by Maisonneuve *et al.* [6]. Briefly, male Sprague Dawley rats (240-270 g) were anesthetized i.p. using 50 mg/kg sodium pentobarbital. Following anesthesia, rats were placed in a supine position, and a light surgical incision was made in the femoral region of the left leg. The femoral vein was isolated, and vehicle (isopropanol) or vehicle plus HD in a concentration of 1, 3, or 6 mg/kg was slowly injected over 3-4 min using a sterile 1-mL syringe. The injection volume was 600-650 µL/kg body weight. The incision was closed using 5.7-mm staples. Rats were allowed to recover and had full access to food and water. Rats showed no signs of an adverse reaction to the surgical procedure during the study. At 3, 6, or 24 hr after HD exposure, rats were euthanatized with 100% $CO_2$, and the left lung was dissected, rinsed in normal saline, and snap-frozen in liquid nitrogen.

*BALF Protein/LDH Measurements.* Following euthanasia, an incision was made to expose the trachea. A small incision was made in the trachea, and a 16-gage needle, one and a half inches long with the bevel ground off, was inserted into the trachea and tied into position with surgical silk. The left lung was tied off, surgically removed and immediately frozen in liquid nitrogen for future analysis. The right lung and trachea were lavaged 5 times with 4 ml of 0.9% saline. Bronchoalveolar lavage (BALF) was analyzed for protein and lactate dehydrogenase, (LDH). Protein concentration was analyzed using the method of Lowry *et al.* [14]. LDH was measured in an Hitachi Chemistry Analyzer model 704 (Roche Diagnostics, Indianapolis, IN). All reagents for diagnostic measurements were from Sigma Chemical Co. (St Louis, MO).

*Nuclear Protein Extraction.* The procedure is essentially that of Blackwell *et al.* [13]. Left lung samples were harvested from rats that had been exposed intravenously to HD. Samples were placed into a liquid nitrogen-cooled mortar and reduced to a fine powder with a liquid nitrogen-cooled pestle. Samples were transferred to a Dounce homogenizer that contained a homogenization buffer [pH 7.9] (0.6% IGEPAL, 150 mM NaCl, 10 mM HEPES, 1 mM EDTA, 0.5 mM PMSF, and 1 mM DTT). After incubation in the homogenization buffer, samples were Dounced releasing the nuclei, then centrifuged at 1000 x g for 1 min to pellet cell debris. Supernatants were centrifuged at 3,600 x g for 15 min at 4 C. The nuclei-containing pellets were suspended in a high-salt homogenization buffer [pH 7.9] (25% glycerol, 20 mM HEPES, 420 mM NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM DTT, 5 µg/mL pepstatin A, 5

μg/mL leupeptin, and 5 μg/mL aprotinin) and incubated on ice for 30 min with intermittent vortexing. After centrifugation at 17,900 x g for 5 min at 4 C, supernatants were combined with three volumes of a high-glycerol, low-salt buffer [pH 7.9] (8 mM HEPES, 10% glycerol, 0.1 mM EDTA, 25 mM KCl) before storage at –70 C. Protein concentrations were determined using the BCA Method.

*Preparation of the NF-kB Oligonucleotide Probe.* A synthetic double-stranded oligonucleotide containing a binding site specific for NF-κB was $^{32}$P-labeled using T4 polynucleotide kinase. Unincorporated nucleotides were removed by spin-column chromatography. The labeled probe was quantified and stored at –20 C.

*EMSA Binding Reactions.* Binding reactions were performed at room temperature in the presence of poly (dI-dC)•poly (dI-dC), in a buffer [pH 7.5] (50 mM Tris-HCl, 250 mM NaCl, 5 mM $MgCl_2$, 2.5 mM DTT, 2.5 mM EDTA, and 20% glycerol) containing 10 to 20 μg of nuclear extract. Labeled NF-κB probe was added and the reactions were allowed to proceed on ice for 15 min prior to electrophoresis. Vacuum-dried gels were exposed overnight to X-ray film. Positive control reactions contained commercially produced HeLa nuclear extract. The specificity and performance of the system were demonstrated by performing a competition reaction in which unlabeled NF-κB oligonucleotide was added to a reaction containing HeLa nuclear extract and labeled NF-κB probe.

*Densitometeric Analyses.* Autoradiographs of the NF-κB shifted complexes were scanned with a Bio Rad GS-700 Imaging Densitometer and analyzed using Bio Rad Multi Analyst™ software. For each sample, the volume densities, expressed as the optical density x mm$^2$, for the two complexes were added. Vehicle control samples were evaluated under identical experimental conditions concurrently with samples from HD-exposed animals. Data were standardized and expressed as a fraction of vehicle control response. The fractional response was calculated by dividing the total volume density value for the HD-exposed samples by the average value for the vehicle control samples from the same time point.

*Statistical Analyses.* LDH and protein data were analyzed using a one-way ANOVA. If statistical differences were found, a Dunnet's *post hoc* multiple comparison test was run to test for differences between groups. Statistical analyses were also performed using numerical values obtained from densitometric data. A two-factor analysis of variance (ANOVA) model was fitted to the NF-κB fractional response data to determine the effects of HD dose and time. Model results indicated that neither HD dose nor the interaction between HD dose and time produced statistically significant effects. Thus, a one-factor ANOVA model was fitted that examined the effect of time. Model parameters from this one-factor model were used to estimate the mean NF-κB fractional response and standard error. For each time, a t-test was used to determine whether the mean NF-κB fractional response differed from one, which would indicate that the response for HD-dosed animals was significantly different from that of control animals. A Bonferroni adjustment for multiple comparisons [15] was applied when evaluating these estimates.

# RESULTS

The data are presented as the summation (1 mg/kg+3 mg/kg+6 mg/kg) of the effects of all doses at each time point. There were no dose-response effects of HD, but we did observe that the overall responses were higher at various study time points.

Analysis of BALF for LDH, an indicator of cell/tissue damage, was determined over all doses of HD at each time point as mentioned above. These data revealed a significant increase over vehicle control at 24 h, $p<0.013$, compared with all other experimental groups (Figure 1).

Intravenous exposure to HD showed that there was an increase in the amount of protein lavaged from the lungs (Figure 2). There appeared to be a slight temporal increase, but this difference was only significant at the 24-h point, $p\leq0.05$, compared with IPA control rats and 3 and 6 h groups.

NF-κB consists of protein dimers that typically produce two sequence-specific gel shifted complexes, a p50/p50 homodimer and a p50/p65 heterodimer, that are visible on an autoradiograph. Overall results indicate that NF-κB levels were not increased in rat lung tissues from animals euthanatized 3, 6, or 24 hr following i.v. exposure to 1, 3, or 6 mg HD/kg body weight (Figures 3-14). Statistical analyses did indicate a minor reduction in NF-κB binding proteins in samples taken from animals euthanatized at 24 hr (mean ± SE NF-κB fraction of vehicle control = 0.84 ± 0.032; $p<0.001$) due to minor reductions in NF- κB response among animals exposed to 3 and 6 mg HD/kg body weight exposure.

# DISCUSSION

These studies were conducted to investigate the premise that the redox sensitive transcription factor NF-κB is involved in mediating the inflammatory response that occurs after exposure to sulfur mustard. Most genes contain promotors or enhancers within their regulatory elements that contain binding sites for multiple transcription factors, including NF-κB. NF-κB is normally present in the cytosol in a non-DNA-binding form. Extracellular signals lead to its translocation into the nucleus where it can bind to gene promoter and/or enhancer sequences and activate the expression of genes involved in the inflammatory and immune response [12, 16, 17]. Acute pulmonary inflammation, such as is caused by HD inhalation, involves the sloughing of tracheal and bronchial epithelial cells that line the respiratory tract [3]. These cell types may initiate an inflammatory response through the release of mediators that increase vascular permeability and attract blood-borne inflammatory cells. The edematous response leads to reduced pulmonary function. The influx of inflammatory cells into the exposed tissue may produce additional damage through the release of reactive oxygen species, proteases, and other inflammatory mediators. There is evidence that vesicant-induced pulmonary toxicity in rodents is mediated via oxidative stress mechanisms, and elevations in antioxidant enzyme activities, lipid peroxidation, and oxidized glutathione--all known inducers of free radical-mediated oxidative stress--have been reported [8].

It is readily apparent that there is a latent exposure–response effect of HD treatment using the BALF sampling technique on the integrity of the air-blood barrier. The most surprising data show that intravenous HD administration is capable of affecting the permeability of the lung. Protein levels are significantly higher at 24 h compared with all other time points (Figure 2). The

latent result of systemic pulmonary toxicity is supported by the fact the BALF LDH is also significantly increased at this time (Figure 1).

Results indicate that, at the concentrations of HD and time points evaluated, alterations in the level of NF-κB do not mediate HD-induced pulmonary inflammation in rodents intravenously exposed to HD. Neither the interaction between HD dose and time nor the HD dose effect was shown to be statistically significant.

This lack of effect could be related to the well-known fact the HD can form DNA adducts through alkylation processes. Langenburger *et al.* [18] have shown that intravenous administration of HD in guinea pigs caused significant DNA-adduct formation. The primary target organ for formed adducts was the lung with the earliest formation occurring within 3 min of injection and continuing on out to 96 h. This suggests that the main target of intravenous HD is the lung. It is therefore reasonable to assume that the failure of increasing doses of HD to activate NF-κB may be related to the rapid formation of DNA adducts that may interfere with NF-κB regulated transcription processes in the presence of HD-mediated lung injury.

In relationship to the initial set of questions posed in the Introduction, this study has provided information that HD given by the intravenous route does cause lung injury as shown in Figures 1 and 2. However, regarding questions 2 and 3, we demonstrate that NF-κB binding proteins in lung tissue of HD-exposed rats are not up-regulated, suggesting that alterations in the level of NF-κB do not mediate HD-induced pulmonary inflammation in rodents intravenously exposed to HD. Therefore, we surmise that it is unlikely that oxidative stress, as measured by the up-regulation of NF-κB, directly causes HD-induced lung injury. Although NF-κB was not found to be predictive of HD-induced pulmonary damage, the literature does indicate that activated NF-κB may be predictive of other causes of toxicant-induced pulmonary damage [13].

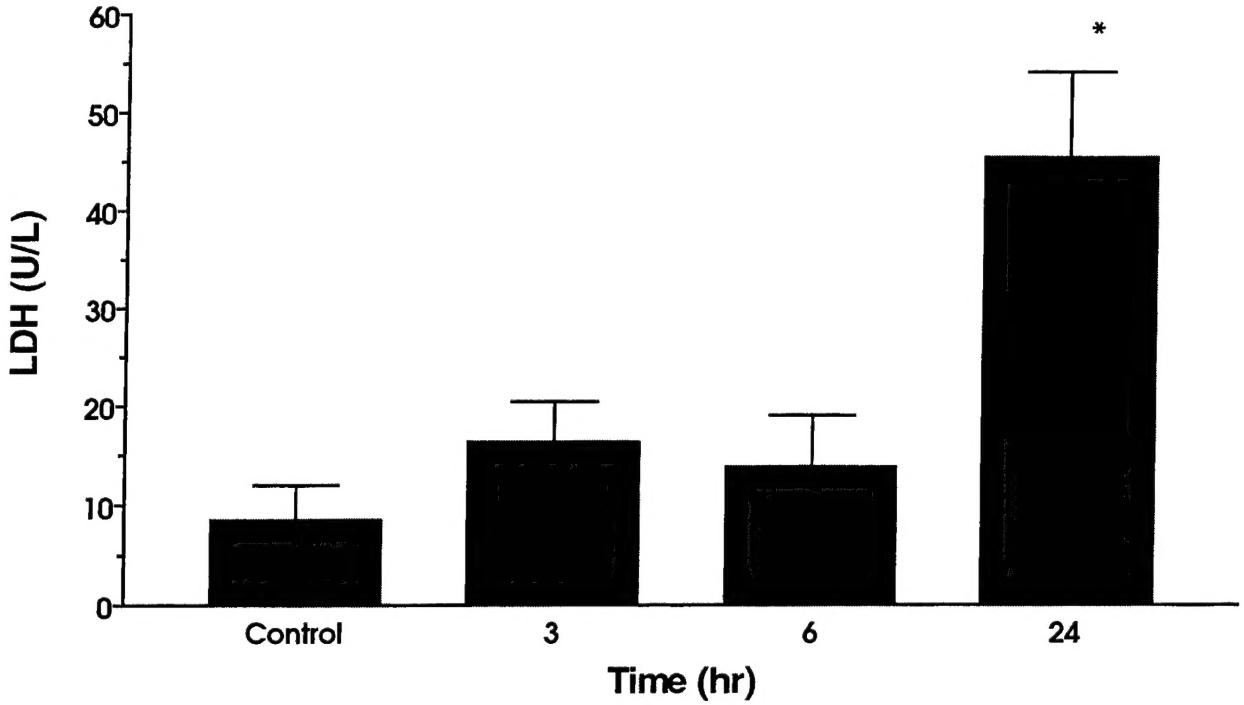


**Figure 1.** Analysis of BALF for lactate dehydrogenase, LDH, over all doses of mustard at each time point in rats exposed to mustard, revealed a significant increase over vehicle (IPA) controls at 24 hours, $p<.013$(*). Values are means +/- SE.

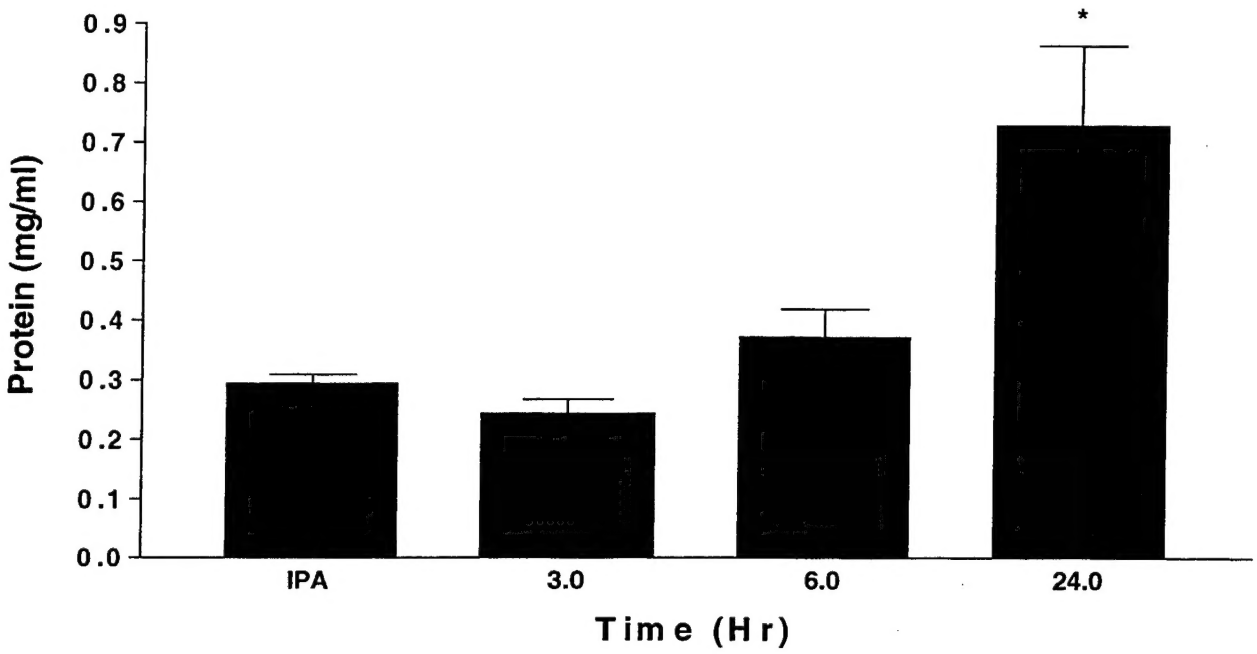


**Figure 2.** Analysis of protein over all doses of mustard at each time point showed an increase in protein over the 3 time points with the 24-hour time point being significant over IPA controls, $p<.05$ (*).


**Figure 3.** Autoradiograph of NF-κB shifted complexes for IPA (Lanes 1-5) and 1 mg/kg HD (Lanes 6-10) exposed samples. Animals were euthanatized 3 hr following exposure.

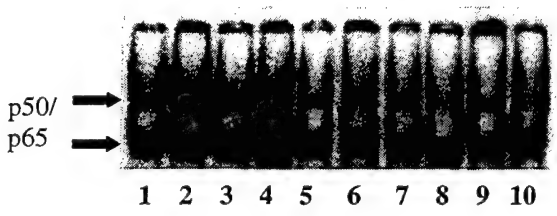


**Figure 4.** Autoradiograph of NF-κB shifted complexes for IPA (Lanes 1-5) and 3 mg/kg HD (Lanes 6-10) exposed samples. Animals were euthanatized 3 hr following exposure.

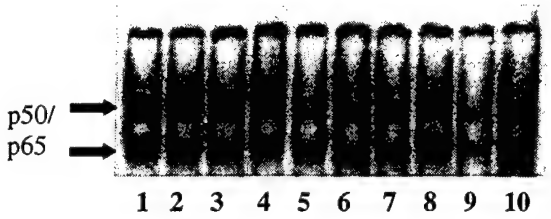


**Figure 5.** Autoradiograph of NF-κB shifted complexes for IPA (Lanes 1-5) and 6 mg/kg HD (Lanes 6-10) exposed samples. Animals were euthanatized 3 hr following exposure.

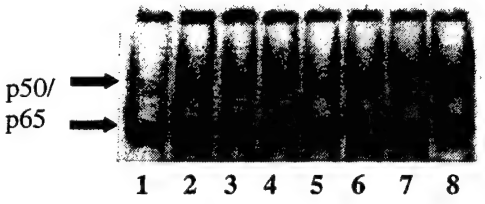


**Figure 6.** Autoradiograph of NF-κB shifted complexes for IPA (Lanes 1-4) and 1 mg/kg HD (Lanes 5-8) exposed samples. Animals were euthanatized 6 hr following exposure.

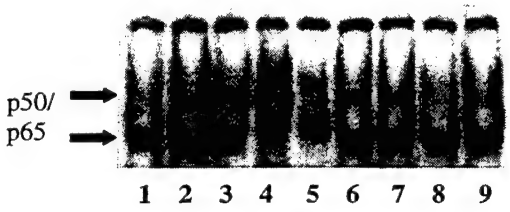


**Figure 7.** Autoradiograph of NF-κB shifted complexes for IPA (Lanes 1-4) and 3 mg/kg HD (Lanes 5-9) exposed samples. Animals were euthanatized 6 hr following exposure.

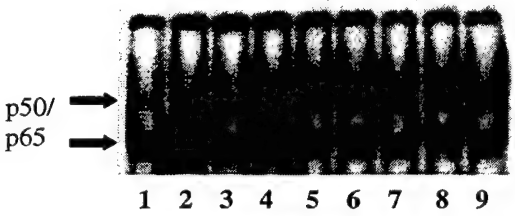


**Figure 8.** Autoradiograph of NF-κB shifted complexes for IPA (Lanes 1-4) and 6 mg/kg HD (Lanes 5-9) exposed samples. Animals were euthanatized 6 hr following exposure.

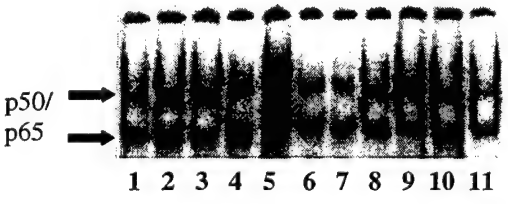


**Figure 9.** Autoradiograph of NF-κB shifted complexes for IPA (Lanes 1-5) and 1 mg/kg HD (Lanes 6-11) exposed samples. Animals were euthanatized 24 hr following exposure.

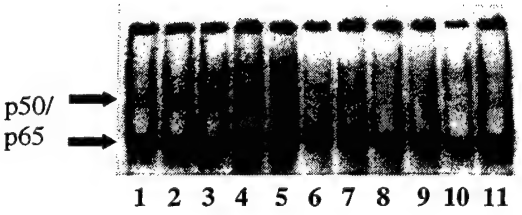


**Figure 10.** Autoradiograph of NF-κB shifted complexes for IPA (Lanes 1-5) and 3 mg/kg HD (Lanes 6-11) exposed samples. Animals were euthanatized 24 hr following exposure.


**Figure 11.** Autoradiograph of NF-κB shifted complexes for IPA (Lanes 1-5) and 6 mg/kg HD (Lanes 6-11) exposed samples. Animals were euthanatized 24 hr following exposure.

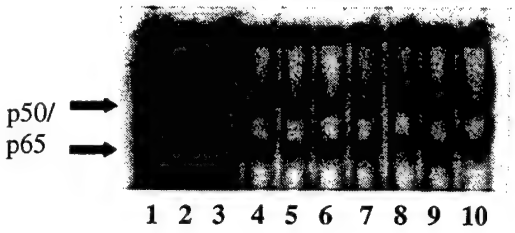


**Figure 12.** Autoradiograph of NF-κB shifted complexes for HeLa extract control (Lane 1), specific competition reaction that included 1.75 pmol non-radioactive double-stranded NF-κB oligonucleotide in the binding reaction (Lane 2), non-specific competition reaction that included 1.75 pmol non-radioactive double-stranded AP-2 oligonucleotide in the binding reaction (Lane 3), IPA (Lane 4), 1mg/kg HD (Lanes 5 and 6), 3 mg/kg HD (Lanes 7 and 8), and 6 mg/kg HD (Lanes 9 and 10) exposed samples. Animals were euthanatized 3 hr following exposure.

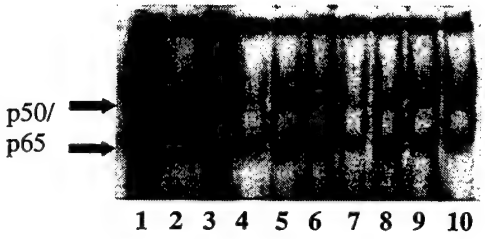


**Figure 13.** Autoradiograph of NF-κB shifted complexes for HeLa extract control (Lane 1), specific competition reaction that included 1.75 pmol non-radioactive double-stranded NF-κB oligonucleotide in the binding reaction (Lane 2), non-specific competition reaction that included 1.75 pmol non-radioactive double-stranded AP-2 oligonucleotide in the binding reaction (Lane 3, IPA (Lane 4), 1mg/kg HD (Lanes 5 and 6), 3 mg/kg HD (Lanes 7 and 8), and 6 mg/kg HD (Lanes 9 and 10) exposed samples. Animals were euthanatized 6 hr following exposure.

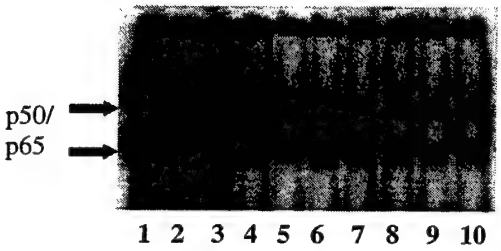


**Figure 14.** Autoradiograph of NF-κB shifted complexes for HeLa extract control (Lane 1), specific competition reaction that included 1.75 pmol non-radioactive double-stranded NF-κB oligonucleotide in the binding reaction (Lane 2), non-specific competition reaction that included 1.75 pmol non-radioactive double-stranded AP-2 oligonucleotide in the binding reaction (Lane 3), IPA (Lane 4), 1mg/kg HD (Lanes 5 and 6), 3 mg/kg HD (Lanes 7 and 8), and 6 mg/kg HD (Lanes 9 and 10) exposed samples. Animals were euthanatized 24 hr following exposure.

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